

# The Role of Environmental Ammonia in Respiratory Mycoplasmosis of Rats

J. Roger Broderson, DVM, MS, J. Russell Lindsey, DVM, MS, and James E. Crawford, BS

Young adult, pathogen-free rats of Sherman and Fischer (F344) substrains were inoculated intranasally with  $10^8$  colony-forming units (CFU) of *M. pulmonis* and housed for 4 to 6 weeks in environments with ammonia maintained at specific concentrations from 25 to 250 ppm. All levels of  $\text{NH}_3$ —whether produced naturally from soiled bedding or derived from a purified source—significantly increased the severity of the rhinitis, otitis media, tracheitis, and pneumonia (including bronchiectasis) characteristic of murine respiratory mycoplasmosis (MRM). The prevalence of pneumonia, but not that of other respiratory lesions of MRM, showed a strong tendency to increase directly with environmental  $\text{NH}_3$  concentration. In contrast,  $\text{NH}_3$  exposure of rats not infected with *M. pulmonis* caused anatomic lesions that were unlike those of MRM and were limited to the nasal passages. It was concluded that environmental  $\text{NH}_3$ , at concentrations commonly encountered in present day cage environments for rats, plays an important role in pathogenesis of MRM. (Am J Pathol 85:115-130, 1976)

FOR MANY YEARS it has been known that pure cultures of *Mycoplasma pulmonis* given intranasally to rats regularly cause the rhinitis, otitis media, and tracheitis, but not the lung lesions, of natural murine respiratory mycoplasmosis (MRM). This observation apparently led several earlier investigators<sup>1-3</sup> to conclude that a "virus" was responsible for the pulmonary lesions of MRM, a belief still prevalent in the United States. Although a few laboratories<sup>4-7</sup> recently have shown that all essential clinical and pathologic features of natural MRM can be reproduced in the rat by *M. pulmonis*, such trials have not consistently reproduced the pneumonia and more advanced lung lesions (bronchiectasis and pulmonary abscesses) commonly associated with this agent in contemporary rat stocks.<sup>8</sup> In essence then, the enigma of why experimental reproduction of the pulmonary disease in MRM has been so elusive remains as perhaps the most crucial problem in elucidating the natural history of this disease.

Circumstantial evidence from a variety of sources<sup>8-10</sup> suggests that some environmental factor(s) may be a major determinant of pathogenic events associated with *M. pulmonis* infection in the rat. Ammonia

---

From the Birmingham Veterans Administration Hospital and the Department of Comparative Medicine, University of Alabama Medical Center, Birmingham, Alabama.

Supported in part by an in-service fellowship to Dr. Broderson from the US Public Health Service.

The studies reported herein were conducted in animal facilities accredited by the American Association for the Accreditation of Laboratory Animal Care.

Accepted for publication June 8, 1976.

Address reprint requests to Dr. J. Russell Lindsey, Department of Comparative Medicine, University of Alabama Medical Center, Birmingham, AL 35294.

(NH<sub>3</sub>) was selected for initial investigation as it is the most common and abundant noxious gas identified in animal facilities.<sup>11</sup> The purpose of the present study was to test the hypothesis that levels of NH<sub>3</sub> normally encountered in rat cages play a contributing role in pathogenesis of the lung lesions in MRM.

## Materials and Methods

### Animals

Two- to four-month-old rats of Sherman (Animal Breeding and Holding Unit, Center for Disease Control, Lawrenceville, Ga.) and Fischer (F344) (rearing described previously<sup>5</sup>) substrains were used, experimental and control groups being matched according to age, sex, and number (5 or 6 rats) per cage. Both substrains were shown to be pathogen free (PF) on repeated culturing for fungi, mycoplasmas, and bacteria and on serologic testing (Center for Disease Control, Atlanta, Ga. and Microbiological Associates, Inc., Bethesda, Md.) for rodent viruses (sendai, reovirus 3, pneumonia virus of mice, Kilham rat virus, Theiler's GD VII, polyoma, ectromelia, lymphocytic choriomeningitis, mouse hepatitis virus, adenovirus, K virus, minute virus of mice, Toolan H-1, and rat corona virus).

### Ammonia Exposure

Details of the experimental design are given in Table 1. In Experiments I and II, rats experimentally infected with *M. pulmonis* were housed in shoebox cages. The bedding of high NH<sub>3</sub> groups was not changed in order to permit natural production of NH<sub>3</sub> by action of bacteria on excreta. Controls (low NH<sub>3</sub>) were provided with fresh bedding daily.

In Experiments III-VII, rats infected with *M. pulmonis* were exposed to NH<sub>3</sub> from a pure source. Gaseous NH<sub>3</sub> was dispensed from the vapor phase of a 150-pound tank of liquid anhydrous NH<sub>3</sub> (PB & S Chemical Co., Henderson, Ky.) through a specially designed flow module to filter and regulate the flow of NH<sub>3</sub> into the entering air stream of an exposure chamber (modified Trexler type plastic film isolator measuring 60 inches long, 24 inches high, and 24 inches wide).<sup>12</sup> Controls (low NH<sub>3</sub>) were housed in a similar isolator not supplied with NH<sub>3</sub>. Experimental (high NH<sub>3</sub>) and control rats were maintained in suspended wire cages over bedding changed daily to prevent natural NH<sub>3</sub> production. To further diminish NH<sub>3</sub> levels in control chambers, the turnover of air was increased by reducing the thickness of outflow filters and/or adding a second air pump.

As additional controls, uninfected PF rats were exposed to NH<sub>3</sub> alone, either produced by soiled bedding in shoebox cages (Experiment VIII) or delivered from the tank of liquid NH<sub>3</sub> (Experiment IX).

### Animal Maintenance

Rats were housed in two types of cages: a) For experiments employing NH<sub>3</sub> evolved naturally from bedding, standard shoebox cages (polycarbonate: 6½ inches deep, 12 inches wide, and 14 inches long) were filled to a depth of ¼ inch with bedding and maintained either inside exposure chambers (Experiments I and II) or outside (Experiment VIII) covered with polyester filters (Filter Bonnets, Filtek, Appleton, Wisc.). b) For studies where rats were exposed to purified NH<sub>3</sub> (Experiments III-VII, IX), special stainless steel cages 7½ inches deep, 16 inches long, and 9¾ inches wide were constructed of ½ inch wire mesh and mounted on legs 6 inches high over trays filled to a depth of ¼ inch with bedding. Hardwood chip bedding (Isodri, Carworth Farms, Inc., New City, NY) was used for both types of cage.

Table 1—Design of Experiments

Experiment No.	No. rats*	NH <sub>3</sub> levels (mean $\pm$ SD) (ppm)	Exposure chamber air turnover	Type caging	NH <sub>3</sub> source	Days preinoculation NH <sub>3</sub> exposure†	Days postinoculation NH <sub>3</sub> exposure‡
I (Sh)	23 24	11 $\pm$ 19 108 $\pm$ 39	6/hr 3/hr	Shoebox	Bedding	7	35
II (F)	24 22	2 $\pm$ 6 212 $\pm$ 31	8/hr 3/hr	Shoebox	Bedding	7	30
III (F)	12 12	Trace‡ 25 $\pm$ 5	10/hr 3/hr	Wire	Tank	7	42
IV (F)	12 12	Trace 25 $\pm$ 4	10/hr 3/hr	Wire	Tank	7	30
V (F)	12 12	Trace 50 $\pm$ 10	6/hr 3/hr	Wire	Tank	7	28
VI (F)	12 12	Trace 100 $\pm$ 12	6/hr 3/hr	Wire	Tank	7	28
VII (F)	12 12	Trace 250 $\pm$ 6	6/hr 3/hr	Wire	Tank	7	28
VIII (Sh)	10 10	10 $\pm$ 18 150 $\pm$ 35	—§ —	Shoebox§	Bedding	75	—¶
IX (F)	12 12	Trace 250 $\pm$ 8	6/hr 3/hr	Wire	Tank	35	—¶

SD = standard deviation, Sh = Sherman strain rat, F = Fischer strain rat.

\* Control (low NH<sub>3</sub>) over experimental groups.† Days rats maintained at desired NH<sub>3</sub> level before and after inoculation with *Mycoplasma pulmonis*.

‡ Indicates levels only rarely detectable, never exceeding 5 ppm.

§ Rats housed in filter-covered cages, all others in cages within plastic film exposure chambers.

¶ Uninfected controls, not given *M. pulmonis*.

Rats were fed autoclaved diet formulated for axenic rodents (Allied Mills or Purina) and given sterile tap water *ad libitum*.

#### Environmental Measurements

Levels of  $\text{NH}_3$  in animal rooms and cages were monitored daily by use of  $\text{NH}_3$  detectors of Honeywell (Minneapolis, Minn., model UVH-10c, range 0 to 200 ppm) and Kitagawa (Unico, Environmental Instruments, Fall River, Mass., range 0 to 700 ppm) types.<sup>12</sup> The latter method also was used in daily monitoring cage levels of carbon dioxide ( $\text{CO}_2$ ), oxygen ( $\text{O}_2$ ), hydrogen sulfide ( $\text{H}_2\text{S}$ ), and sulfur dioxide ( $\text{SO}_2$ ). All samples of cage air were taken 1 inch above the bottom of the cage (bedding or wire). Temperature and relative humidity in rooms and exposure chambers were measured with standard laboratory thermometers and sling psychrometers, respectively.

Air turnover in exposure chambers was determined by placing a flow meter over the exhaust port and calculating flow rate versus volume of the isolator. Smoke generating capsules (Smoke Sticks, Hill, Inc., San Francisco, Calif.) were used in determining turnover rate and dispersion of incoming air within isolators.

#### *M. pulmonis* Inoculations

The inoculum was a broth culture (second artificial passage of "culture A" described previously<sup>8</sup>) of *M. pulmonis*, identified as a pure culture by immunofluorescence<sup>13</sup> and found to contain  $8 \times 10^8$  colony-forming units (CFU) of *M. pulmonis* per 0.05 ml.<sup>5</sup> Following preexposure of rats to the desired level of  $\text{NH}_3$  for 7 days, fentanyl and droperidol (Innovar-Vet, Pitman-Moore, Inc., Washingtons Crossing, NJ) were given (1:10 dilution in distilled water; 0.1 ml/rat intraperitoneally) to induce the catatonic state during which each rat was given 0.05 ml of the inoculum intranasally.

#### Culture Techniques

*Mycoplasma* broth was made, using PPLO broth (Difco, Detroit, MI) without crystal violet, to contain the following: 0.05% glucose; 10% yeast extract; 20% horse serum (BBL); penicillin, 500 units/ml; and thallium acetate 1:2000. Phenol red was added as an indicator, and the final pH was adjusted to 7.8.

Cultures were taken aseptically at necropsy. Each trachea was perfused in the midcervical region with 0.5 ml of phosphate-buffered saline using an 18-gauge needle on a tuberculin syringe. Each aspirate was used to inoculate 3 ml of mycoplasma broth, 3 ml of brain-heart infusion broth, and blood agar plates.

#### Pathology

At the termination of experiments, each rat was given 30 mg of pentobarbital sodium intraperitoneally. When complete analgesia was achieved, rats were exsanguinated by incising the femoral vessels in the groin and collecting blood for virus serology. Representative sera collected at this time were consistently negative upon serologic testing for rodent viruses (listed above).

The methods of collecting and processing tissues of the respiratory tract for histopathology have been reported previously.<sup>8</sup> Sections were coded and evaluated as "unknowns," with microscopic findings at each level of the tract (nasal passages, middle ears, tracheas, and lungs) being scored (0 to 3) according to the severity of the lesions previously described for MRM.<sup>5</sup> The sum of scores for each organ divided by the sum of maximum scores possible gave the lesion index for making comparisons of experimental groups. A lesion index of 1.0 was the most severe change possible for each organ.

Histologic sections also were prepared from liver, kidney, adrenal, pancreas, testicle, spleen, mediastinal nodes, and thymus.

### Statistics

For comparisons of nonparametric data such as the lesion index, Wilcoxon's two-sample rank test<sup>14</sup> was used. The chi-square method was used for data giving only "present or absent" information.<sup>15</sup> Regression and correlation analysis were used to test for relationships between increasing environmental  $\text{NH}_3$  concentrations and prevalence of lesions or frequency of *M. pulmonis* isolations.

## Results

### Effects of Natural $\text{NH}_3$ on Murine Respiratory Mycoplasmosis

#### Environmental Data

Experiments I and II were conducted as preliminary studies to explore the effects which  $\text{NH}_3$  produced naturally in soiled bedding might have on pathogenesis of MRM. Rats inoculated with *M. pulmonis* were housed on bedding in shoebox cages within plastic film isolators. Except for  $\text{NH}_3$  levels (Table 1), environmental conditions did not differ significantly between those isolators in which cages were supplied fresh bedding daily and those in which the bedding was not changed. The environmental conditions (mean  $\pm$  SD) were  $\text{CO}_2$ ,  $0.13 \pm 0.04\%$ ; temperature,  $23.6 \pm 0.5^\circ\text{C}$ ; and relative humidity,  $69 \pm 5.0\%$ . In these and all subsequent experiments,  $\text{H}_2\text{S}$  and  $\text{SO}_2$  were not present at detectable levels (0.005% and 0.02%, respectively).

#### Clinical

Typical signs of MRM, including snuffling and sporadic head shaking, began in rats of all groups around 10 days postinoculation. Moderate dyspnea and hyperpnea occurred in a few animals in the high  $\text{NH}_3$  groups beginning on Day 14. Rats exposed to high levels of  $\text{NH}_3$  had rough hair coats and sat in a hunched posture.

#### Pathology

All lesions observed were typical of MRM.<sup>5</sup> In both Experiments I and II, gross and microscopic lung lesions were observed more frequently in high  $\text{NH}_3$  groups than in controls (Table 2). Atelectasis and consolidation occurred in rats of both high  $\text{NH}_3$  and low  $\text{NH}_3$  groups, but bronchiectasis was seen only in rats exposed to high  $\text{NH}_3$  (108 and 212 ppm).

Microscopic evaluation of lesions at each level in the respiratory tract, (i.e., nasal passages, middle ears, trachea, and lungs) revealed quantitative differences between high  $\text{NH}_3$  and control groups in both experiments (Table 3). The differences were statistically significant ( $P < 0.05$ ) except for otitis in Experiment I.

Table 2—Prevalence of Lung Lesions in Rats Inoculated With *Mycoplasma pulmonis* and Exposed to Ammonia

Exp. No.	Experimental NH <sub>3</sub> level (ppm)	Gross lesions		Microscopic lesions	
		High NH <sub>3</sub>	Low NH <sub>3</sub>	High NH <sub>3</sub>	Low NH <sub>3</sub>
I	108	20/24 (83%)*	10/23 (43%)	23/24 (96%)*	14/23 (61%)
II	212	6/22 (27%)†	2/24 (8%)	19/22 (86%)*	8/24 (33%)
III	25	5/12 (42%)	1/12 (8%)	9/12 (75%)	6/12 (50%)
IV	25	6/12 (50%)	4/12 (33%)	9/12 (75%)	7/12 (58%)
V	50	8/12 (66%)‡	2/12 (17%)	10/12 (83%)‡	9/12 (75%)
VI	100	4/12 (33%)	1/12 (8%)	12/12 (100%)	6/12 (50%)
VII	250	10/12 (83%)	2/12 (17%)	12/12 (100%)	2/12 (17%)

\*  $P < 0.01$ ; chi-square test comparing high and low NH<sub>3</sub> groups of Experiments I and II.

†  $P < 0.1$ ; chi-square test comparing high and low NH<sub>3</sub> groups of Experiment I.

‡  $P < 0.01$ ; chi-square test comparing summed data of high NH<sub>3</sub> groups with summed data of low NH<sub>3</sub> groups in Experiments III through VII.

#### Culture Results

*M. pulmonis* was recovered from a significantly larger proportion of rats in the high NH<sub>3</sub> group in Experiment I ( $P < 0.05$ ), but not in Experiment 2 (Table 4). No pathogenic bacteria other than *M. pulmonis* were isolated.

#### Effects of Purified NH<sub>3</sub> on Murine Respiratory Mycoplasmosis

##### Environmental Data

NH<sub>3</sub> concentrations in the exposure chambers of the high NH<sub>3</sub> groups in Experiments III–VII were relatively stable throughout the exposure periods (Table 1). In the control chambers, NH<sub>3</sub> only occasionally reached detectable levels and never exceeded 5 ppm. Other environmental parameters were not significantly different between the two chambers, or between the chambers and the room environments. Mean CO<sub>2</sub> was  $0.10 \pm 0.03\%$ . The mean temperature was 23.0 to 24.0 C ( $\pm 0.4$  to  $\pm 1.0$  C), except in Experiment VII, which was conducted during a time of mechanical problems with air conditioning, resulting in a mean temperature of 25.1 C ( $\pm 0.9$  C). Mean relative humidities ranged between 51 and 69% ( $\pm 4.4$  to  $\pm 12.9\%$ ) for different experiments.

##### Clinical

No differences were observed during the preconditioning periods before inoculations. Signs of respiratory disease following inoculations were similar to those observed in rats of Experiments I and II. Upon removal of animals from the exposure chambers at termination of the experiments,

Table 3—Severity of Microscopic Lesions in Rats Inoculated With *Mycoplasma pulmonis* and Exposed to Ammonia

Exp. No.	Mean NH <sub>3</sub> Level (ppm)*	Lesion index			
		Rhinitis	Otitis	Tracheitis	Pneumonia
I	11	.09†	.52	.49‡	.29†
	108	.65	.71	.85	.69
II	2	.13†	.28†	.54†	.15‡
	212	.77	.77	.89	.44
III	Trace	.00	.24‡	.50‡	.22†
	25	.06	.58	.89	.64
IV	Trace	.56‡	.39‡	.86	.31‡
	25	.97	.86	.94	.53
V	Trace	.75‡	.50‡	.92	.31†
	50	1.00	.81	.92	.63
VI	Trace	.39†	.50‡	.78‡	.25†
	100	1.00	.89	1.00	.67
VII	Trace	.31†	.00†	.33†	.06†
	250	1.00	.53	1.00	.83

\* Control (low NH<sub>3</sub>) over experimental groups.

†  $P < 0.01$ ; Wilcoxon's two-sample rank test comparing control and experimental values.

‡  $P < 0.05$ ; Wilcoxon's two-sample rank test comparing control and experimental values.

the characteristic snuffling of MRM was much more pronounced in the high NH<sub>3</sub> groups than in controls.

#### Pathology

All lesions observed were characteristic of MRM.<sup>5</sup> Grossly, there were marked differences between high NH<sub>3</sub> groups and controls. Rats in the high NH<sub>3</sub> groups had more cervical lymphadenopathy than controls.

Table 4—Recovery of *Mycoplasma pulmonis* From Inoculated Rats Exposed to Ammonia

Exp. No.	Experimental NH <sub>3</sub> level (ppm)	No. of positive cultures/total No. of cultures	
		High NH <sub>3</sub>	Low NH <sub>3</sub>
I	108	19/24 ( 79%)*	11/23 (48%)
II	212	18/22 ( 82%)	22/24 (92%)
III	25	11/12 ( 92%)	8/12 (67%)
IV	25	ND	ND
V	50	11/12 ( 92%)†	11/12 (92%)
VI	100	12/12 (100%)	4/12 (33%)
VII	250	12/12 (100%)	0/12 ( 0%)

ND = No data due to overgrowth of cultures by bacteria other than *M. pulmonis*.

\*  $P < 0.05$ ; chi-square test comparing high and low NH<sub>3</sub> groups in Experiments I and II.

†  $P < 0.01$ ; chi-square test comparing summed data of high NH<sub>3</sub> groups with summed data of low NH<sub>3</sub> groups in Experiments III through VII.

Gross bronchiectasis and/or pulmonary abscesses (Figure 1A-D) were observed in lungs from 8 to 33% of rats in high  $\text{NH}_3$  groups compared to none or 8% of controls. Without exception, prevalence (Table 2) and extent of gross atelectasis and consolidation were greater in rats exposed to high  $\text{NH}_3$ . Prevalence of microscopic lung lesions characteristic of MRM also was greater in high  $\text{NH}_3$  groups.

The data on prevalence of lung lesions in all high  $\text{NH}_3$  groups were summed and compared by the chi-square test with the sum of data for the corresponding control groups (Table 2). Differences between the high  $\text{NH}_3$  and control groups in prevalence of gross and microscopic lung lesions were highly significant ( $P < 0.01$ ).

Regression analysis was used to determine whether the increasing  $\text{NH}_3$  concentrations in Experiments III-VII were associated with incremental changes in prevalence of lung lesions. When all groups of high  $\text{NH}_3$  rats (25 to 250 ppm) were considered by regressing percentage of rats with lesions (Table 2) against the log of their respective  $\text{NH}_3$  concentrations, increasing concentrations of  $\text{NH}_3$  were positively related to increasing prevalence of gross ( $P < 0.09$ ) and microscopic ( $P < 0.06$ ) lesions. Also, increasing  $\text{NH}_3$  concentration was positively correlated with increasing gross ( $r = .6$ ) and microscopic ( $r = .9$ ) lesions.

With rare exceptions, the severity of microscopic lesions in nasal passages, middle ears, tracheas, and lungs was significantly ( $P < 0.05$ ) greater in high  $\text{NH}_3$  groups (Table 3).

#### Culture Results

Although the results were not consistent, *M. pulmonis* generally was cultured more frequently from rats in  $\text{NH}_3$  groups of Experiments III-VII (Table 4). When the sum of the data for high  $\text{NH}_3$  groups was compared by chi-square analysis with the sum of the data for control groups, the difference was highly significant ( $P < 0.01$ ). However, increasing  $\text{NH}_3$  concentration was not associated with an increasing frequency of *M. pulmonis* isolations (regression analysis,  $P > 0.05$ ; coefficient of correlation,  $r = 0.1$ ).

#### Effects of $\text{NH}_3$ Without Murine Respiratory Mycoplasmosis

##### $\text{NH}_3$ From Contact Bedding

Uninoculated Sherman rats were divided into two groups and housed in shoebox cages with filter caps (Experiment VIII). Cages in the control group were changed three times weekly, while the high  $\text{NH}_3$  group had litter partially replaced on occasion but never completely changed. The

mean  $\text{NH}_3$  concentrations in the cages were  $\text{NH}_3$  group,  $150 \pm 35$  ppm, and controls,  $10 \pm 18$  ppm (Table 1).  $\text{CO}_2$  concentrations were slightly higher than in open cages, the mean being  $0.18 \pm 0.05\%$ . Relative humidity and temperatures were not recorded.

After 1 week, when the  $\text{NH}_3$  level had reached 100 ppm, the rats showed excessive blinking of the eyes. Control rats were normal in appearance and behavior throughout the study.

#### Purified $\text{NH}_3$

Uninoculated Fischer rats were housed in suspended wire cages inside the high  $\text{NH}_3$  and control chambers used in previous studies (Experiments III–VII) of *M. pulmonis* infection and exposure to  $\text{NH}_3$ . Experimental and control chambers differed only in  $\text{NH}_3$  concentration (Table 1). The means of other environmental conditions were  $\text{CO}_2$ ,  $0.10 \pm 0.03\%$ ; temperature,  $23.7 \pm 0.05^\circ\text{C}$ ; and relative humidity,  $66 \pm 5.0\%$ .

No clinical difference was observed in appearance of rats exposed to  $\text{NH}_3$  and those of the control group.

#### Pathology

Pathologic changes were found in the nasal passages of all rats exposed to high  $\text{NH}_3$  from bedding (Experiment VII) or the purified source (Experiment IX). None was seen in control rats. Lesions were most extensive anteriorly, where all except the ventral one-fourth to one-third of the nasal passages was affected. Progressing posteriorly, lesions gradually decreased in the dorsoventral dimension, with only a very small zone of the dorsal-most recesses of olfactory mucosa being involved at the cribriform plate. Along the lower margin of this affected zone, from external nares to cribriform plate, there was a sharp boundary between diseased and normal mucosa.

Histologic alterations were the same in both respiratory and olfactory epithelium. The epithelium was increased in thickness to three or four times normal. In affected areas, many epithelial cells along the basement membrane had pyknotic nuclei and were distended by moderately eosinophilic cytoplasm (Figures 3 and 4). A few such cells were scattered singly in the submucosa nearby. In some fields there was hyperplasia of epithelial cells with formation of glandular crypts. A few neutrophils were seen in the epithelial layer, the lumina of submucosal glands, and the nasal passages.

The submucosa in the affected zone was widened by marked dilatation of small vessels and edema, giving the connective tissue stroma a delicate honeycombed appearance. In rats exposed to  $\text{NH}_3$  for 35 days or longer,

there was replacement of submucosal glands by collagen (Figure 3). A few lymphocytes and neutrophils were present.

### Discussion

Although possible influences of environmental factors on pathogenesis of MRM have been suspected for many years,<sup>8-10</sup> the present study is the first to provide experimental evidence that such a factor does indeed play a significant role in the natural history of this important disease. Exposure of *M. pulmonis*-infected rats to NH<sub>3</sub> at concentrations commonly encountered in contemporary cage environments was found to a) increase the rate of isolating *M. pulmonis* from the respiratory tract, b) enhance the severity of the lesions characteristic of MRM throughout the respiratory tract, and c) increase the prevalence of the lung lesions characteristic of MRM. Furthermore, it was demonstrated that increasing NH<sub>3</sub> concentration (in the range of 25 to 250 ppm) was positively correlated with increasing prevalence of lung lesions of MRM in *M. pulmonis*-infected rats.

The significance of these findings is best appreciated in the context of the relationship between infection and disease due to *M. pulmonis*. This agent is ubiquitous as an infection of the upper respiratory tract in conventionally reared laboratory rats throughout the world.<sup>5</sup> Numerous investigators<sup>4-6,16</sup> have found that upper respiratory infection and disease are readily established by experimental intranasal inoculation of rats with pure cultures of *M. pulmonis*. Likewise, in the present studies essentially every rat infected with *M. pulmonis* (high NH<sub>3</sub> and control groups) developed microscopic rhinitis, otitis media, and tracheitis and the organism usually was isolated from the trachea in a high proportion of animals regardless of exposure to NH<sub>3</sub>. In both the natural and experimental infection, the lung lesions of MRM occur inconsistently but always in association with upper tract disease and/or infection due to *M. pulmonis*.<sup>5,8</sup>

Thus, it appears that *M. pulmonis* under normal circumstances preferentially inhabits the upper respiratory tract and produces lung disease secondarily. Perhaps chronic exposure to NH<sub>3</sub>, through injury to the nasal mucosa, indirectly enhances growth of *M. pulmonis* in the upper tract and, consequently, provides a larger inoculum for the lung. The effects of NH<sub>3</sub> also may be mediated through impaired clearance of the agent from the respiratory tract as Dalhamn<sup>17</sup> has reported that environmental concentrations of NH<sub>3</sub> as low as 3 ppm were effective in causing ciliostasis in rat trachea. Also, environmental NH<sub>3</sub> may directly enhance the growth of *M. pulmonis* in the respiratory tract of rats. Any or all of these potential

mechanisms appear possible based on the unique anatomic lesions we observed in nasal passages due to  $\text{NH}_3$  alone.

The findings of the present work also have important implications to the longstanding controversy about the etiology of the lung lesions in MRM.<sup>5</sup> Our data support the thesis originally advanced by Klieneberger and Steabben<sup>18</sup> that *M. pulmonis* is the infectious agent responsible for natural MRM in the rat and raise further doubts about a contributing role by a "virus" as some have postulated.<sup>1-3</sup>

Although deserving of far greater attention, the literature contains few controlled studies in which  $\text{NH}_3$  from natural sources has been shown to be detrimental to an animal's health.  $\text{NH}_3$  in poultry houses has been incriminated in causing keratoconjunctivitis,<sup>19</sup> reduced weight gains,<sup>20</sup> and increased susceptibility to respiratory infections<sup>21-23</sup> in birds. Doig and Willoughby<sup>24</sup> reported mild thickening of nasal and tracheal epithelium of pigs exposed to 100 ppm  $\text{NH}_3$  for 2 to 6 weeks.

The results of the present studies more closely parallel those of Sato *et al.*<sup>25</sup> reported for *Mycoplasma gallisepticum* in the chicken. In their study, chicks were inoculated intratracheally with *M. gallisepticum* and exposed to 20, 50, or 100 ppm  $\text{NH}_3$  for up to 28 days.  $\text{NH}_3$  at each of these concentrations dramatically increased both the numbers of *M. gallisepticum* recovered from trachea and the severity of respiratory tract lesions due to the mycoplasma.

With few exceptions, most of the attention given  $\text{NH}_3$  levels in animal facilities in the past has been focused on concentrations in the macroenvironment (room) environment inhabited by personnel rather than on the microenvironment (cage) occupied by experimental animals. Briel *et al.*<sup>11</sup> have shown that animal numbers, frequency of cage sanitation, and ventilation are important factors influencing cage levels of  $\text{NH}_3$ . Serrano<sup>26</sup> observed that it is not unusual for mouse cages to exceed 100 ppm  $\text{NH}_3$  under normal conditions or 200 ppm when crowded and covered with filters or other materials which impede circulation. Murakami<sup>27</sup> reported levels of  $\text{NH}_3$  up to 40 ppm in mouse cages.

Similar data have not been reported for the rat. However, a brief survey of rat cages in a modern animal facility during the course of the present studies revealed frequent measurements of  $\text{NH}_3$  concentration above 50 ppm. The threshold limit value (i.e., maximum allowable concentration for man over an 8-hour work day) in the United States is 25 ppm,<sup>28</sup> the lowest concentration tested and found to enhance MRM in the present study. We also have observed that measurements made at the level of the rat's nose (1 to 2 inches above bedding) generally give much higher  $\text{NH}_3$  concentrations than air samples taken farther away from soiled bedding.

Thus, it is imperative when considering cage concentrations of  $\text{NH}_3$  to think in terms of the stratum of air which represents the true micro-environment of the rat.

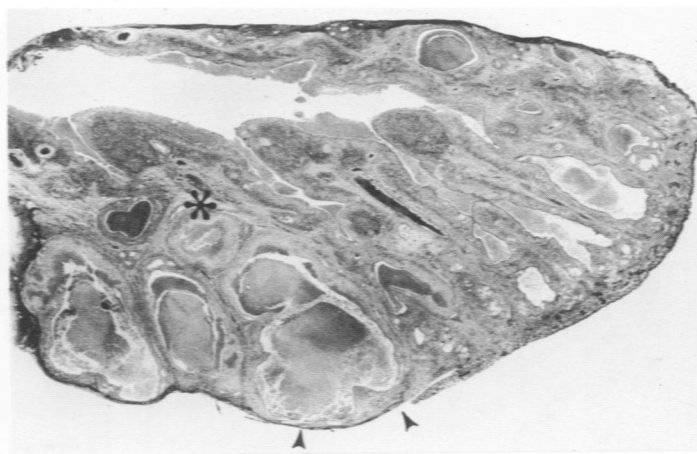
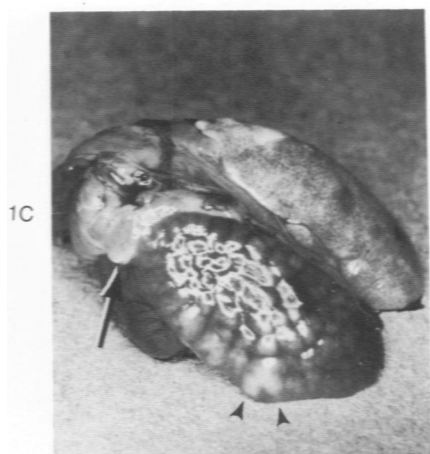
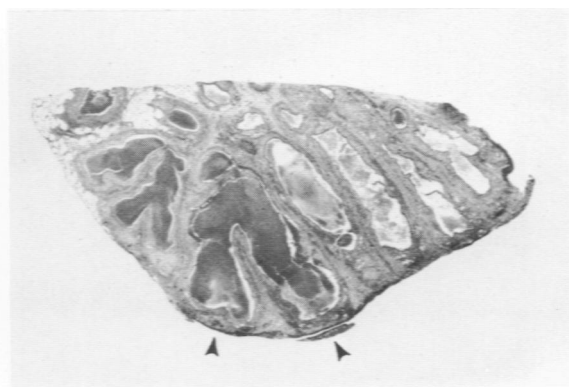
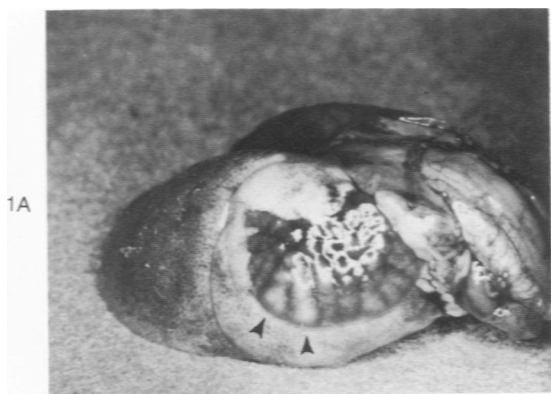
The present data support the contention that the microenvironment of laboratory rats is often seriously polluted by  $\text{NH}_3$  and this, in turn, enhances dramatically the ubiquitous murine respiratory mycoplasmosis in this species. Further studies will be required to determine the mechanisms responsible and to answer the equally intriguing question of whether environmental  $\text{NH}_3$  has other effects on laboratory rats and data from experiments employing this species.

### References

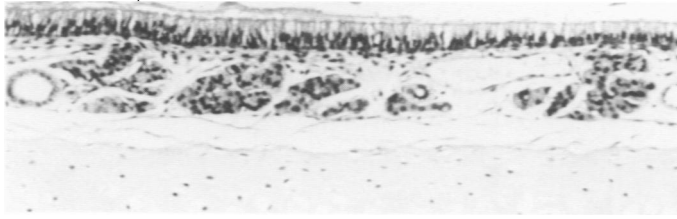
1. Nelson JB: The etiology and control of chronic respiratory disease in the rat. Proc Anim Care Panel 7:30-40, 1957
2. Vrolijk H, Verlinde JD, Braams WG: Virus pneumonia (snuffling disease) in laboratory rats and wild rats due to an agent probably related to grey lung virus of mice. *Antonie van Leeuwenhoek* 23:172-183, 1957
3. Joshi N, Dale DG, Blackwood AC: Etiology of murine endemic pneumonia. *Rev Can Biol* 24:169-178, 1965
4. Kohn DF, Kirk BE: Pathogenicity of *Mycoplasma pulmonis* in laboratory rats. *Lab Anim Care* 19:321-330, 1969
5. Lindsey JR, Baker HJ, Overcash RG, Cassell GH, Hunt CE: Murine chronic respiratory disease: Significance as a research complication and experimental production with *Mycoplasma pulmonis*. *Am J Pathol* 64:675-716, 1971
6. Whittlestone P, Lemcke RM, Olds RJ: Respiratory disease in a colony of rats. II. Isolation of *Mycoplasma pulmonis* from the natural disease, and the experimental disease induced with a cloned culture of this organism. *J Hyg (Camb)* 70:387-409, 1972
7. Jersey GC, Whitehair CK, Carter GR: *Mycoplasma pulmonis* as the primary cause of chronic respiratory disease in rats. *J Am Vet Med Assoc* 163:599-604, 1973
8. Cassell GH, Lindsey JR, Overcash RG, Baker HJ: Murine mycoplasma respiratory disease. *Ann NY Acad Sci* 225:395-412, 1973
9. Lane-Petter W, Olds RJ, Hacking Mr, Lane-Petter ME: Respiratory disease in a colony of rats. I. The natural disease. *J Hyg (Camb)* 68:655-662, 1970
10. Giddens WE Jr, Whitehair CK, Carter GR: Morphologic and microbiologic features of trachea and lungs in germfree, defined-flora, conventional and chronic respiratory disease-affected rats. *Am J Vet Res* 32:115-129, 1971
11. Briel JE, Kruckenberg SM, Besch EL: Observations of Ammonia Generation in Laboratory Animal Quarters. Institute for Environmental Research, Kansas State University, Manhattan, Kansas, Feb 1972
12. Broderson JR, Lindsey JR, Crawford JE: A method for exposure of rats to controlled low levels of ammonia gas. (Unpublished data)
13. Del Guidice RA, Robillard NF, Carski TR: Immunofluorescence identification of *Mycoplasma* on agar by use of incident illumination. *J Bacteriol* 93:1205-1209, 1967
14. Snedecor GW, Cochran WG: Statistical Methods, Sixth edition. Ames, Iowa, Iowa State University Press, 1971, pp 555-556
15. Cochran WG: Some methods for strengthening the common  $\chi^2$  tests. *Biometrics* 10:417-451, 1954
16. Nelson JB: The nasal transmission of pleuropneumonia-like organisms in mice and rats. *J Infect Dis* 82:169-176, 1948

17. Dalhamn T: Mucous flow and ciliary activity in the trachea of healthy rats and rats exposed to respiratory irritant gases. (SO<sub>2</sub>, H<sub>2</sub>N, HCHO): A functional and morphologic (light microscopic and electron microscopic) study, with special reference to technique. *Acta Physiol Scand* 36(Supp 123):1-161, 1956
18. Klieneberger E, Steabben DB: On the association of the pleuropneumonia-like organism L3 with bronchiectatic lesions in rats. *J Hyg* 40:223-227, 1940
19. Carnaghan RBA: Keratoconjunctivitis in broiler chicks. *Vet Rec* 70:35-37, 1958
20. Charles DP, Payne CG: The influence of graded levels of atmospheric ammonia on chickens. II. Effects on the performance of laying hens. *Br Poult Sci* 7:189-198, 1966
21. Anderson DP, Beard CW, Hanson RP: The adverse effects of ammonia on chickens including resistance to infection with newcastle virus. *Avian Dis* 8:369-379, 1964
22. Anderson DP, Wolfe RR, Cherms FL, Roper WE: Influence of dust and ammonia on the development of air sac lesions in turkeys. *Am J Vet Res* 29:1049-1058, 1968
23. Valentine H: A study of the effect of different ventilation rates on the ammonia concentration in the atmosphere of broiler houses. *Br Poult Sci* 5:149-159, 1964
24. Doig PA, Willoughby RA: Response of swine to atmospheric ammonia and organic dust. *J Am Vet Med Assoc* 159:1353-1361, 1971
25. Sato S, Shoya S, Kobayashi H: Effect of ammonia on *Mycoplasma gallisepticum* infection in chickens. *Nat Inst Anim Health Q (Tokyo)* 13:45-53, 1973
26. Serrano LJ: Carbon dioxide and ammonia in mouse cages: Effect of cage covers, population, and activity. *Lab Anim Sci* 21:75-85, 1971
27. Murakami H: Differences between internal and external environments of the mouse cage. *Lab Anim Sci* 21:680-684, 1971
28. The American Conference of Government Industrial Hygienists: Documentation of Threshold Limit Values for Substances in Workroom Air. Cincinnati, Ohio, 1973, p 10

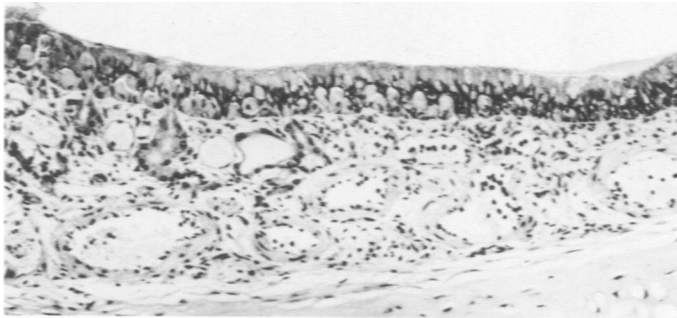
*[Illustrations follow]*



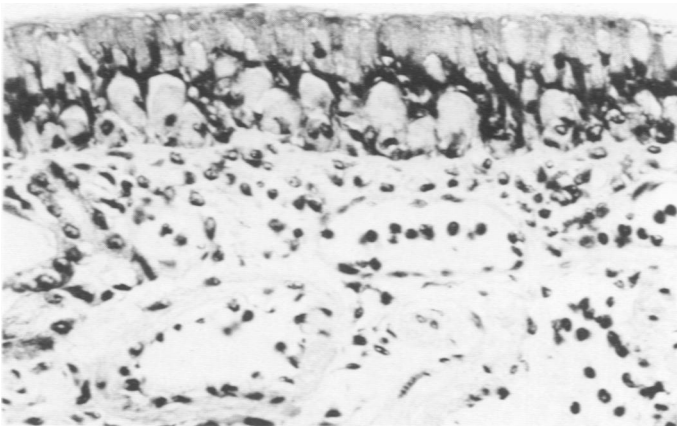
**Figure 1**—Lung from a rat given *M. pulmonis* and exposed to 25 ppm  $\text{NH}_3$  for 42 days. Gross and microscopic lesions are demonstrated, respectively, in the right cranial lobe (A and B) and in the left lobe (C and D). *Small arrows* indicate matching bronchioles in gross and microscopic specimens. The lung lesions present include bronchiectasis, bronchiolectasis, and true abscesses (resulting from accumulation of purulent exudate and destruction of bronchiolar epithelium) such as the one indicated (*asterisk*). Also, an enlarged lymph node is present anterior (*large arrow*) to the left lobe in C. (A,  $\times 2.0$ ; C,  $\times 1.5$ ; B and D, H&E,  $\times 7.0$ )



**Figure 2**—Nasal septum of control pathogen-free rat exposed to trace levels of  $\text{NH}_3$  (H&E,  $\times 100$ ).



**Figure 3**—Nasal septum of pathogen-free rat exposed to 150 ppm  $\text{NH}_3$  for 75 days. The mucosa and submucosa are greatly thickened. Epithelial cells are variably rounded and distended, resulting in loss of normal cell pattern. The submucosa shows depletion of glands, dilatation of vessels, and minimal chronic inflammation. (H&E,  $\times 100$ )



**Figure 4**—Higher magnification of Figure 3 (H&E,  $\times 260$ ).